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Review

Airway-to-biophase transfer of inhaled oligonucleotides

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Abstract

Because systemic delivery of proteins and peptides proceeds via the lung, and oligonucleotides (ONs) possess some similar molecular characteristics, it is tempting to propose that inhalation may solve some of the systemic delivery problems being experienced by scientists in the ON industry. It is also likely that a variety of ON targets exist within the lung itself. Although the lungs and respiratory tract (RT) may offer their own metabolic challenges, this route offers an enormous absorptive surface area, quite capable of slowly delivering 5–7 kDa compounds to the circulation. Moreover, the presentation of formulated ONs appears capable of suppressing the local (lung) expression of gene products involved in inflammation and other disease processes. In this review we describe the current state of the art surrounding the cell biology of ONs and then critically analyze those factors which determine the feasibility of their delivery by aerosol inhalation. At present, systemic dosing requirements for the majority of ONs are a little too large to envisage their routine delivery via inhalation. ON molecular design to increase potency and decrease susceptibility to nuclease enzymes may or may not change this situation in the near future. On the other hand, ON delivery for the purposes of inhibiting expression of proteins involved with lung pathology at a local level is advocated because it appears to be within easy reach of current aerosol delivery technology.

Keywords: Inhaler; Pulmonary delivery; Aerosol; Inhalation dosimetry; Airway-to-biophase transfer; Oligonucleotide; Transcription suppression; Immunomodulator; Receptor; Macromolecule; Absorption; Lung clearance

Contents

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1. Introduction

Pulmonary drug delivery by aerosol is presently receiving considerable attention. Our knowledge of the processes affecting respiratory disease is increasing, a fact which is leading to the development of a large number of new chemical entities with local (lung) therapeutic activities [1]. Difficulties associated with the systemic delivery of macromolecules, coupled with the likelihood of their reasonable pulmonary bioavailabilities, have led to interest in their presentation for absorption via the lung [2]. Finally, the requirement that the industry replace the ozone-depleting chlorofluorocarbon propellants presently used in many inhalers has initiated a rapid expansion in the available aerosol delivery options [3–5].

ONs are a new group of macromolecular compounds with seemingly endless applications related to their ability to modify the expression of genes within cells [6]). While ultimately ONs must penetrate the cell membrane and reach intracellular targets in order to exhibit their activities, cellular targets for aerosol therapy could be either inside (locally active ONs) or outside (systemically active ONs) the lung. While this article is intended to cover a number of areas, one of its primary purposes will be to discuss the feasibility of ON delivery as therapeutic or prophylactic agents in inhalation aerosols. Specifically, we will seek to answer the following questions:

1. What are the maximum drug doses which can be administered by aerosol inhalation?
2. How do systemic and local doses for ONs compare to these values?
3. What are the speed and molecular size limits for pulmonary ON absorption?
4. Can local (lung) ON activities be enhanced by formulation maneuvers?
5. Are there aerosol formulation and generation problems associated with ONs?

To add perspective to these 'drug delivery questions' we have added a review of the important cell and molecular biology literature surrounding ONs, especially where this literature prevents or confuses our arrival at a clear answer to any of the questions above. We have added recent data from our own laboratory where this was relevant to the discussion. Finally, we have selected an ON from the literature as a case study for inhalation feasibility assessment to illustrate some of our more important points and to identify specific areas for further research.

2. On cell biology

2.1. Inhibition of gene expression

2.1.1. Mechanism of action

ONs are a new class of chemical compounds which are currently being tested in clinical trials as antiviral and anti-thrombin agents. These single strands of DNA (deoxyribonucleic acid) are usually 12 to 25 nucleotides in length and specifically block expression of target genes. DNA ONs are most commonly used although RNA (ribonucleic acid) ONs may also be used

but suffer from the disadvantage of being considerably less stable in vivo and in vitro. A gene is the DNA blueprint by which cells make a specific cellular protein. All cellular proteins are encoded by their respective DNA sequence (gene) including structural proteins such as actin, or enzymes which execute all cell functions. The process of gene expression involves many steps beginning with the double-stranded DNA in the cell's nucleus. Structurally, each DNA strand is a polymer made of a sequence of nucleotides. Each nucleotide has a deoxyribose sugar attached to one of four bases: A (adenine), G (guanine), C (cytosine), and T (thymine) (Fig. 1). Phosphate groups join the nucleotides by connecting one ribose sugar at the C5 carbon to the next sugar at the C3 carbon. Two DNA strands form a 'double helix' based upon hydrogen binding between complementary bases, i.e. A binds T, C binds G.

In gene expression, one strand of the DNA in the double helix acts as a template for synthesis of hnRNA (heteronuclear ribonucleic acid) (Fig. 2). This process, called transcription, is also based on base-pairing rules, i.e. A:T, C:G, U:A (the RNA equivalent of T is U, uracil). Then, in a process called splicing, the RNA molecule is processed to remove non-coding sequences, called introns. The resulting mRNA sequence (messenger RNA) containing only exons, or coding sequences, is transported to the cytoplasm where a ribosome binds to the mRNA and translates RNA base triplets into the corresponding amino acid which is added to the growing polypeptide (protein) chain in a process called translation (Fig. 2).

Each of the major steps involved in gene expression has been tested as targets for ONs. Transcription, RNA processing, and RNA translation have all been successfully blocked by ONs

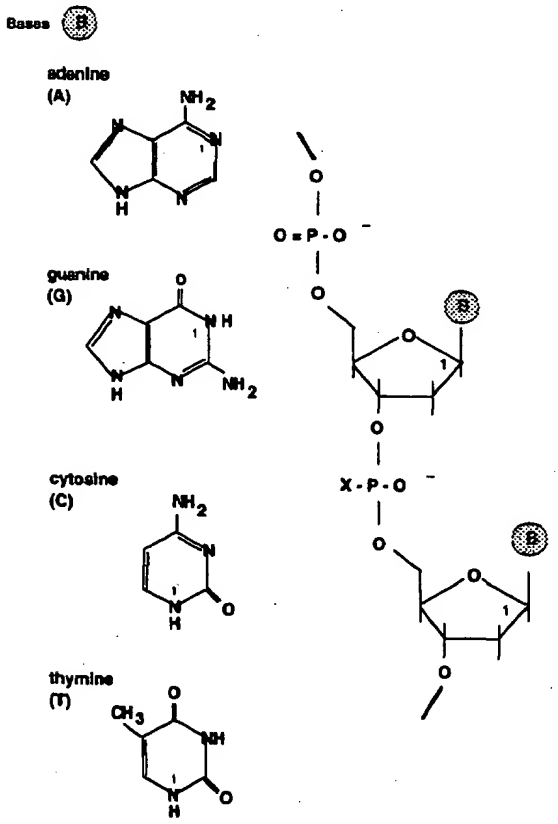

Fig. 1. The structure of ONs. ONs are polymers made of deoxyribose sugars attached via a phosphate backbone. A glycosidic bond attaches the C1 of the sugar to the base (B), i.e. the N9 of adenine or guanine, or the N1 of cytosine or thymine. X indicates common sites of ON backbone modification. X=sulfur in phosphorothioate ONs, X=CH_3 in methylphosphonate ONs.

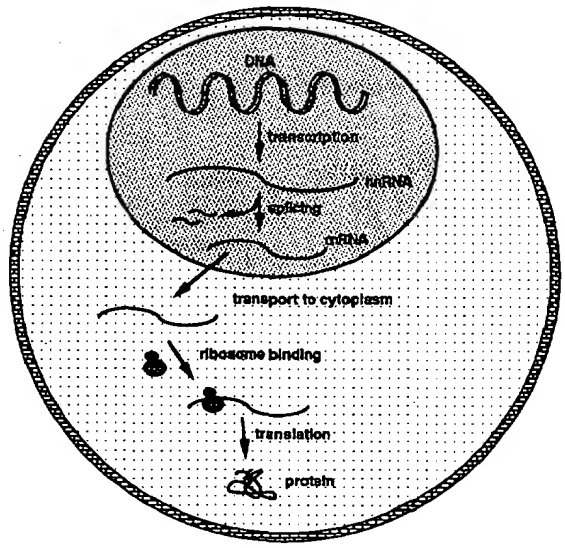

Fig. 2. Gene expression. Nuclear DNA is transcribed into hnRNA. Non-coding regions of the hnRNA are removed, resulting in mRNA. mRNA is transported into the cytoplasm where the sequence is translated by ribosomes into the gene product (protein).

resulting in specific inhibition of gene expression. For example, triplex-forming ONs bind specific regions of double-stranded DNA and thereby specifically impair transcription of the target gene (Fig. 3A). Early work in this area demonstrate ON triple helix formation with DNA: pyrimidine-rich (T and C) ONs bind DNA via Hoogsteen base-pairing [7] while purine-rich (A and G) ONs bind DNA via reverse Hoogsteen base-pairing [8]. Triplex-forming sequences have been used to regulate gene expression via triple helix formation in live cells [9–11]. Another successful approach involves the use of double-stranded ONs which act as competitive inhibitors for DNA binding and transcription factors resulting in inhibition of DNA replication or RNA synthesis [12].

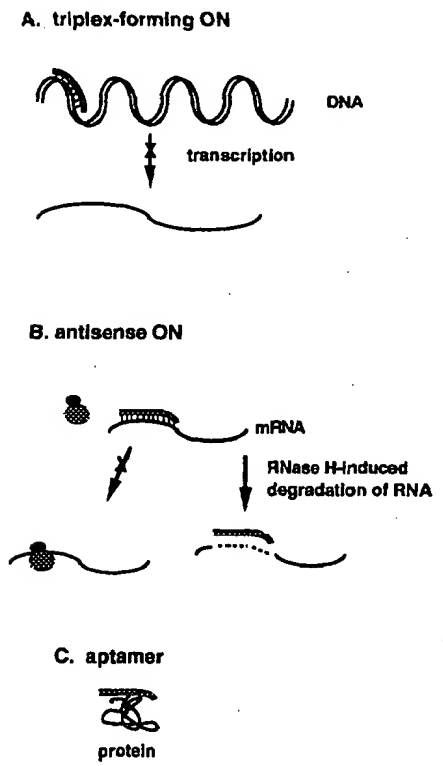

Fig. 3. Mechanisms of ON action. (A) ONs may bind double-stranded DNA resulting in triple helix formation and inhibition of RNA synthesis. (B) ONs may bind mRNA resulting in inhibition of ribosome binding or degradation of complementary RNA via RNase H. (C) ONs may also be designed to bind proteins with high specificity.

Antisense ONs are designed to bind to complementary RNA sequences via Watson-Crick base-pairing rules, i.e. TA, GC, and AU base pairs (Fig. 3B). ONs which bind the translation initiation region or adjacent areas inhibit translation initiation [13,14] (Fig. 3B); ONs targeted to the RNA splice regions inhibit RNA processing [15]; and ONs which bind mRNA coding [16] or non-coding [17] regions may result in termination of protein synthesis. One mechanism ONs block protein synthesis is via the enzyme RNase H (ribonuclease H) (Fig. 3B). RNase H degrades RNA when duplexed to single-stranded DNA [18,19]. Therefore, in addition to interfering with the translation or splicing machinery, ONs may also induce degradation of complementary RNAs via RNase H. The latter mechanism is used by unmodified ONs and some ON derivatives including phosphorothioate ONs [20] (Fig. 1).

While ON therapy resembles gene therapy in that both treatments involve the use of DNA molecules, the two technologies differ significantly (Table 1). The primary difference lies in the function of the administered DNA. In gene therapy, the DNA eventually encodes the pharmacological agent, a protein. In contrast, in ON therapy, the ON itself is the pharmacological agent. However, recombinant DNA may also be used to express antisense RNA sequences which can bind to complementary RNA targets and specifically block gene expression. In this case, the DNA mediates the delivery of the pharmacological agent, antisense mRNA, to the cell. This approach is frequently used in the laboratory to produce prolonged gene down-regulation and has also been especially valuable when used as a tool to study protein function.

The primary obstacle to using recombinant DNA therapeutically is inefficient delivery to cells. DNA is a large, anionic molecule and does not readily enter cells undegraded. To circumvent this limitation, viruses have been used as gene carriers [21]. Briefly, genes required for viral replication are removed, thus rendering the virus replication-incompetent. The desired sequence (which either encodes an antisense mRNA or the protein to be synthesized) is then inserted into the viral genome in lieu of the

Table 1
ON vs. gene therapy

	Estimated total size (kDa)	Size of DNA	Estimated frequency of administration	Recombinant DNA	Potential antigenicity
Free PO[a] ON	5–7	15–25 nucleotides	infusion?	N	low
Free PS[b] ON	5–7	15–25 nucleotides	48 h	N	low
PS ON + cationic lipid	>50	15–25 nucleotides	48 h	N	low
Gene therapy	>10[n]	>35 kilobases	≥biweekly	Y	high

Therapeutic considerations of gene and some ON therapies are compared. Data presented are estimates from the literature.
[a]Phosphodiester ON
[b]Phosphorothioate ON.

Table 2
Properties of three major ON backbone derivatives

ON[a]	Backbone	Charge at pH 7	Stereo-isomer	Effectiveconcentration concentration (μM)[b]	Solubility	Purification	Availability	Purity	Stability (cells)	Stability (in vivo)
PO	$^{-}$O-P=O	negative	N	5–100	freely soluble in H_2O	HPLC or gel	excellent	excellent	minutes/hours	minutes
PT	$^{-}$S-P=O	negative	Y	0.5–20	freely soluble in H_2O	HPLC or gel	excellent	excellent	hours/days	hours/
MP	CH_3-P=O	neutral	Y	25–100	300 μg/ml	HPLC or gel	poor	excellent	hours/days	minutes

The properties and commercial availability of three first generation ON backbone derivatives are compared. Data presented are estimates of properties reported in the literature.
[a]PO, phosphodiester; PT, phosphorothioate; MP, methylphosphonate.
[b]For review, see [140].

replication genes. The recombinant viral genome is then repackaged into the virus using helper cells (for review see [22]). The helper cell synthesizes large quantities of the altered virus which is infectious, but unable to replicate. Intensive efforts are underway to exploit virally mediated nucleic acid delivery system for gene therapy, especially in the treatment of cystic fibrosis [23].

ONs are also being tested as selective protein inhibitors (aptamer ONs) (Fig. 3C). These high-affinity ligands may be used to bind nucleic acid binding proteins [24,25] or other protein molecules [26,27]. The therapeutic potential of these protein inhibitors show great promise for selective protein deactivation. In fact, a phosphodiester ON aptamer candidate is being tested in clinical trials for its thrombin-inactivating properties ([26] and W.A. Lee, personal communication, Meeting of the American Association of Pharmaceutical Scientists, November 1994).

2.1.2. Activity

ONs selectively inhibit cellular and viral protein expression in cultured cells (for review see [6]). Common ON targets include proto-oncogenes, viral proteins, cytokines, and other cellular proteins. Typical effective ON concentrations range from 5 to 100 μM and depend on the cell line, the ON target, and the ON (Table 2). As a result, antisense ONs are/were in clinical trials as an anti-viral agent (antisense to human papilloma virus and cytomegalovirus), and as immune modulators (antisense to intercellular adhesion molecule) (S.T. Crooke, personal communication, Meeting of the American Association of Pharmaceutical Scientists, November 1994).

The biological activity of ONs in animal models is currently under examination. Successful use of ONs in situ and direct injection intraventricularly and intrahypothalamically into animals has been demonstrated (see [6] for review). In addition, at least three studies to date report specific biological activity of ONs in peripheral tissues after parenteral injection. One example is the regression of fibrosacromas in mice after injection of an antisense NF-κB (a transcription factor), but not control, phosphorothioate ON (dose = 40 μg/g mouse body weight) [28]. This study has at least two implications: first, that NF-κB expression is necessary for the maintenance of the transformed phenotype, and second, that intraperitoneal injection of phosphorothioate ONs may be used to specifically block gene expression in target tissues. Similar success was reported by Burch and Mahan [29] who administered 3 nmol antisense IL-1 receptor phosphorothioate ON by subcutaneous injection in mice. The treatment resulted in an inhibition of neutrophil infiltration in response to IL-1 injection. Finally, an antisense phosphorothioate ON complementary to the bcr-abl gene was injected intravenously into a SCID mouse model for leukemia every day for 9 days [30]. Treatment with the antisense ON (1 mg/day) resulted in disappearance of leukemia cells and bcr-abl mRNA transcripts in mouse tissue and prolonged survival of the mice from 8–13 weeks (control ON-treated and untreated animals) to 8–23 weeks (antisense treated animals). Thus, at least in these initial studies, ON specificity and activity continue to be observed in peripheral tissues. To the best of our knowledge, no reports of ON activity in humans have been published to date.

2.1.3. Selectivity

ON effects on non-target gene expression are expected to be minimal since non-matching sequences will bind the ON with much lower affinity relative to sequences which are 100% complementary. Other therapeutic advantages of sequence-specific inhibition of gene expression may result when targeting mutated or mutating sequences. For example, many cancer chemotherapies affect only dividing cells, whereas ON therapy may be useful in non-dividing cells. Because of these potential advantages combined with sequence specificity, ONs represent a class of potentially powerful new drugs, especially in the treatment of cancer and viral infection. Novel applications for ON therapy are also being tested, including immunomodulation ([31] and S.T. Crooke, personal communication, Meeting of the American Association of Pharmaceutical Scientists, November 1994), anti-thrombin agents [26], behavior modulators [32–34] and inhibitors of neointima formation in injured arteries [35,36].

Despite sequence-specific binding, nonspecific

activity may result for a number of reasons. First, the ON must be targeted to a unique sequence to prevent inadvertent regulation of non-target genes. Second, ONs may bind sequences which are not completely complementary, or homologous (see [37] for review). Third, ON-protein interactions may also account for ON non-specific activity. As discussed above, ONs may be designed to bind with high affinity to target protein molecules. In fact, some phosphorothioate ONs have demonstrated non-sequence dependent anti-HIV activity believed to result from reverse transcriptase inhibition [38]. Finally, non-specific cardiovascular toxicity has been associated with infusion of phosphorothioate ONs in monkeys [39,40] and is believed to result from the polyanionic nature of the ON. Similarly, no toxicity was noted in mice, but short-lived intoxication was noted at 160 mg/kg with phosphodiester ONs, and 640 mg/kg phosphorothioate ONs [41]. In human trials, high doses of phosphorothioate ONs appear to affect coagulation and the complement cascade but appear safe at therapeutic doses (S.T. Crooke, personal communication, Meeting of the American Association of Pharmaceutical Scientists, November 1994).

2.2. ON use in the lung

2.2.1. Possible lung targets

In addition to cancer gene targets, ONs have also been targeted to viral proteins, intercellular adhesion molecules (ICAMs) and other cellular proteins. Such genes may also be targeted by ONs in the treatment of lung disorders. For example, ONs may be used to inhibit replication of viruses which infect the lung (e.g. rhinovirus, respiratory syncitial virus, H. influenzae, parainfluenza). Based on in vitro data for ONs targeted to other viruses, ON down-regulation of viral replication or packaging proteins are expected to result in inhibition of the rates of viral replication (for review see [6]). In fact, studies in cultured lung cells have begun to demonstrate the feasibility of ON use for such purposes (see section 2.2.2).

Novel ON applications in the treatment of acute and chronic lung disorders are likely to emerge in the near future in parallel to the increasingly rapid discovery of the biochemical and genetic causes of lung diseases. Obstructive lung disorders may represent a disease state amenable to ON therapy. For example, asthma affects more than 10 million Americans and is an obstructive lung disorder marked by contraction of airway smooth muscle, increased secretions and thickening of the lung mucosa and basement membranes. Diseases such as pulmonary embolism or anaphylaxis are also associated with airway obstruction and are caused by a combination of increased mucous secretion, inflammation, and smooth muscle contraction. Therefore, ON-based treatment of such pulmonary obstructive diseases can potentially be directed at inhibition of bronchial mucous synthesis, secretion, or smooth muscle contraction. However, generalized inhibition of these processes may not be desirable since many organ systems rely upon such processes for their normal function. Specific targeting to the lung or the prevention of systemic absorption after lung delivery of the ONs would therefore be essential for successful use of such a generalized strategy. In addition, the consequences of long-term, repeated administration of these compounds is unknown and whether tolerance, drug resistance, induction of degradative enzymes, etc. will limit chronic use will require further investigation, although no additional toxicity has been observed with long-term (14 days) use of ONs [41].

An alternative approach which may not necessitate ON targeting may be to inhibit the chemical cascades which mediate smooth muscle contraction and bronchoconstriction. These processes rely upon chemical mediators such as leukotrienes, prostaglandins, slow-reacting substance of anaphylaxis, and histamine (for review see [42]). Mediators of bronchoconstriction, such as prostaglandin D2, trypase, sulfidopeptide leukotrienes and histamine, may also be involved in airway inflammation and hyperresponsiveness. Therefore, other pulmonary disorders which are also associated with lung inflammation, such as chronic obstructive pulmonary disease (COPD), emphysema, chronic bronchitis, bronchiectasis and cystic fibrosis, could also conceivably benefit from ON-induced inhibition of prostaglandin, leukotriene and histamine synthesis, perhaps in combination with other therapies. A number of

enzymes are involved in the biosynthesis of mediators of inflammation and bronchoconstriction and may be useful ON targets. For example, both leukotriene C4 synthase and 5-lipoxygenase are involved in leukotriene C4 biosynthesis; cyclooxygenase is required for prostaglandin biosynthesis. In fact, leukotriene antagonists are being tested in clinical trials for the treatment of asthma; cyclooxygenase inhibitors (indomethacin, for example) have also used to antagonize prostaglandin synthesis, although the use of these products have met with limited success. The role of cytokines such as IL-1 in mediating the inflammation associated with asthma is also currently under investigation and may also eventually evolve as viable ON targets. Therefore, while the general approach of inhibition of mediators of bronchoconstriction and inflammation is currently being tested for non-ON drugs, the use of ON therapeutics in this area remains largely unexplored.

The strategies outlined above represents only a few approaches in using ONs in the treatment of obstructive lung disease. Other approaches using ONs may warrant further consideration. For example, emphysema is associated with protease release from lung leukocytes and macrophages followed by protein degradation in lung tissues. ON-mediated inhibition of protease synthesis in lung leukocyte or macrophages may represent a novel method of impairing the progression of emphysema. Aptamer ONs designed to specifically bind key proteases may also be a viable strategy to treat emphysema. In addition, the lung is an important metabolic organ. Angiotensin activation by angiotensin-converting enzyme occurs in the lung; thus, inhibiting synthesis of angiotensin-converting enzyme may be useful in the treatment of hypertension. The lung also inactivates bradykinin, serotonin, some prostaglandins, norepinephrine, and possibly histamine. To the best of our knowledge, the clinical utility of ON-induced blockade of the inactivation of these compounds has yet to be explored.

2.2.2. ON studies in cultured lung cells

In 1991, an estimated 143 000 deaths occurred due to lung cancer in the United States. In fact, the USA ranks 3rd in the world in the incidence of lung cancer in women, and 13th in the world for lung cancer in men [43]. Predisposing factors include smoking, radiation, asbestos, air pollution, age, scarring, and exposure to certain metals. Lung cancer can be divided into subgroups of either small cell lung cancer, or non-small cell lung cancer based on differences in clinical and biological behavior. Among the genetic alterations implicated in lung cancer include mutations in ras, p53, erbB2, and c-myc [44]. Antisense ONs may be useful in the treatment of primary and metastatic lung cancers by selectively correcting the biochemical consequences of such mutations.

Antisense ON studies in cultured lung cells have focused on the biochemical mechanisms underlying lung cell tumorigenicity, but also represent possible new therapeutic approaches. For example, plasmids expressing antisense urokinase sequences decrease lung tumor formation by metastatic cells [45], antisense SV40-T antigen sequences convert immortalized lung fibroblasts to senescence [46], and antisense K-ras sequences decrease tumorigenicity in lung tumor cells [47–49]. In contrast, plasmids expressing antisense sequences targeted to the tumor suppresser genes, retinoblastoma gene product [50] or p53 [51], result in enhancement of lung tumor growth. These studies have been useful in providing additional evidence to determine the role of each of these proteins in lung cell proliferation and tumorigenicity and suggest possible new treatment venues for these incurable diseases.

ON studies in cultured lung cells have not been limited solely to the elucidation of biological mechanisms. For example, ONs have also been successfully used to inhibit intercellular adhesion molecule-1 (ICAM-1) expression in a human lung cell line [52]. Antisense ICAM ONs are currently being tested to down-regulate ICAM expression in tissue transplant [31], and could potentially be used to prevent lung rejection after transplant.

2.2.3. ON studies in the lung

Currently, the first and only example of use of antisense technology in the lung was reported by Georges et al. [48]. A DNA sequence expressing

an antisense mRNA complementary to the ras proto-oncogene was inserted into the adenovirus genome and packaged into an adenovirus particle. These viruses were instilled intratracheally into nude mice which have been previously inoculated with a human large cell lung carcinoma. The investigators found that 87% of the mice treated with the virus expressing the antisense sequence were tumor-free, while in contrast, 90% of mice treated with virus expressing a control sequence grew tumors. These results suggest that this type of lung carcinoma cell proliferation is ras-dependent and also demonstrates the feasibility of using viruses to deliver viable antisense-expressing DNA to the lung.

2.2.4. ON delivery to lung from blood

Data from in vivo trials have begun to identify some in vivo limitations of ON use. Phosphodiester ONs are rapidly degraded in serum [53,54] and are rapidly cleared from the plasma [55]. Phosphorothioate ONs show improved in vivo stability and in some cases an elimination half-life > 27 h in animals [56,57]. After parenteral administration, both phosphorothioate and phosphodiester ONs are distributed throughout the body and to the lung, but not to the brain [55,58]. Attempts to localize the ON into genital warts by intradermal injection directly into the lesion result in rapid systemic absorption and poor localization in the warts (S.T. Crooke, personal communication, Meeting of the American Association of Pharmaceutical Scientists, November 1994). The lung absorbs < 3% of the total dose [55,56] suggesting that fairly large doses will be required to achieve therapeutic concentrations when delivering ONs to the lung after parenteral administration.

Rapid ON metabolism also presents as an in vivo obstacle. Phosphorothioate ONs are rapidly degraded in the liver [59]. Degradation products are excreted in the urine and feces with > 50% of the administered dose eliminated via expired air [56,58,60]. 24 h after injection, between 15 and 50% of the ON extracted from the kidney and liver was found to be intact. The kidney demonstrated the greatest uptake of the ON with maximum levels at approximately 10 to 15% of the injected dose [55,56,58,60]. Because of the long elimination half-life and apparent in vivo stability, phosphorothioate ONs are dosed every other day in human clinical studies (S.T. Crooke, personal communication, Meeting of the American Association of Pharmaceutical Scientists, November 1994). In vivo toxicity studies demonstrate no adverse effects in rats [57] and mice [30], but acute toxicity was observed in monkeys [39,40]. Whether this toxicity is related to high-dose effects on complement in humans is still unclear (S.T. Crooke, personal communication, Meeting of the American Association of Pharmaceutical Scientists, November 1994). Therefore, because of the rapid plasma distribution of parenterally administered ONs and the observed toxicity in monkeys observed at high doses, drug targeting technology or nonparenteral routes of administration may be necessary to achieve therapeutic concentrations of ON in the lung or other target organs.

In vivo targeting of nucleic acids has enjoyed success in preliminary studies. A DNA complex was designed to bind to liver cells expressing asialoglycoprotein receptors. The complex consisted of an asialoorosomucoid-polylysine conjugate and DNA which associates with the positively charged polylysine by ionic interactions which increased hepatic uptake of DNA from 17% to 85%. The delivered CAT gene was also found to be functional, with CAT activity detected in liver homogenates [61]. A similar approach was used to target ONs in vivo using an asialoglycoprotein-polylysine complex with an antisense albumin ON [62]. The ON rapidly dissociates from the conjugate after injection into the rat, but was nevertheless found to be concentrated in the liver 2–3-fold relative to uncomplexed ON. The activity of the ON was not examined in this study, but provides promising preliminary data for the feasibility of in vivo ON targeting. These in vivo studies were based upon initial trials in cultured cells [61–66] have been successfully used in vitro to increase nucleic acid uptake. ONs conjugated directly to receptor ligands have also been tested. For example, a tripeptide which binds the platelet activating factor receptor was conjugated to an antisense actin ON. The tripeptide-ON conjugate demonstrated decreased uptake compared to an un-

conjugated ON, but may be more cytotoxic compared to tripeptide-control ONs [67]. Interestingly, antibody-targeted liposomes were also successfully used to deliver biologically active antisense RNA molecules [68] or ONs [69] to cells.

Research into non-parenteral routes of administration is still in its infancy. For example, methylphosphonate ONs (Fig. 1) appear to penetrate hairless mouse or cadaver skin when applied topically. The extent of penetration was inversely related to the length of the ON and the number of negative charges. As with other topically applied drugs, the stratum corneum proved to be the primary barrier to ON penetration. The biological activity of such topically applied ONs has not yet been established [70]. In addition, orally administered phosphorothioate and phosphodiester ONs are rapidly degraded resulting in loss of activity (W.A. Lee, personal communication, Meeting of the American Association of Pharmaceutical Scientists, November 1994). Systemic absorption after intraocular administration has also been reported [71]. These in vivo studies cumulatively suggest that future research must logically proceed in one or more of the following directions: (1) testing of new or previously synthesized ON analogs for in vivo stability after oral administration; (2) development of strategies to orally deliver phosphorothioate or phosphodiester ONs while circumventing degradation in the gastrointestinal tract; or (3) development of other non-oral routes of ON administration.

2.3. Other obstacles

2.3.1. Stability

As is characteristic of any newly emerging technology, several obstacles continue to impede ON drug development. One factor limiting ON development includes the limited stability of phosphodiester ONs in cells and in serum [20,53,54]. ON susceptibility to nuclease degradation in vitro has been largely addressed by chemical modifications (for review, see [72]. Numerous ON analogs have been synthesized which demonstrate improved stability towards both exonuclease and endonuclease degradation. Phosphorothioate modification results in drastically improved half-lives both in vivo and in vitro: half-life in cultured cells is usually greater than 24 h [73] and intact ON has been found 24 h after injection in animal tissues (see above). However, a frequent result of chemical modification is a reduction in binding affinity for target strands. Phosphorothioate ONs are perhaps the most widely tested ON analog which are nuclease resistant and are more efficiently internalized compared to unmodified ONs. Because of these improved properties, while retaining adequate, but reduced, binding affinities for target sequences, phosphorothioates are being tested in clinical trials as anti-viral agents and immune regulators ([74] and S.T. Crooke, personal communication, Meeting of the American Association of Pharmaceutical Scientists, November 1994).

2.3.2. Cellular uptake

A second obstacle confronting ON development is cell membrane transport and inefficient cellular uptake [75,76]. ON analogs such as methylphosphonate [77,78] or cholesterol-modified [79,80] ONs have been synthesized to increase ON lipophilicity and ON partitioning into biological membranes. However, as mentioned above, altering the structure of the ON frequently results in some loss of potency or even toxicity ([40,75,81,82] and S.T. Crooke, personal communication, Meeting of the American Association of Pharmaceutical Scientists, November 1994). Considerable variation in the extent of uptake and intracellular distribution is observed between cell and ON types, which further obscures complete definition of the ON uptake mechanism [83–86].

The mechanism of ON uptake is believed to be endocytosis in some cell lines (Fig. 4) [85,87,88]. However, the mechanism by which the ON escapes the endocytic vesicle to the active site in the cytoplasm or nucleus remains unclear. Evidence supporting a calcium-dependent, non-endocytic ON uptake mechanism has also been reported [76,89,90]. Clearly, further delineation of the mechanism of uptake are critical to enable rational design of methods to enhance uptake and targeting to cells.

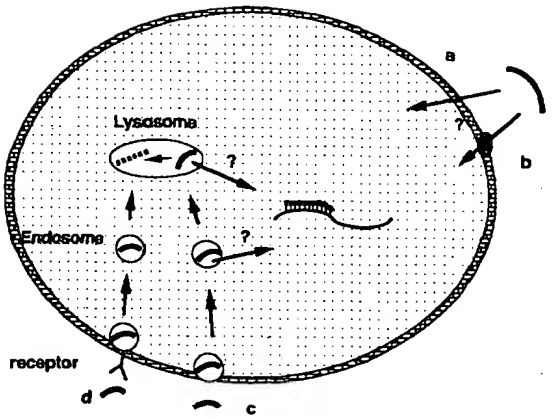

Fig. 4. Known pathways of cell membrane transport. Molecules generally cross the lipophilic cell membrane by one or more of the following mechanisms: (a) diffusion, (b) carrier- or pore-mediated transport, (c) pinocytosis, or (d) receptor-mediated endocytosis. Mechanisms (c) and (d) typically involve either fusion of the endosome with lysosomes or eventual return of the endosome contents to the cell surface (not shown).

Two methods of enhancing the internalization of phosphodiester or phosphorothioate ONs have been tested including use of liposomes or ON modification (for review see [91]). Positively charged lipids, such as DOTMA (*N*-[(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride), have also been successfully used to increase ON uptake and activity [31,92]. For example, Bennet et al. [92] report that cell expression of ICAM-1 (induced by HUVEC cell incubation with interleukin-1β following drug treatment) was only slightly inhibited by treatments with up to 100 μM ISIS 1570. This finding was attributed to poor cellular uptake. Cell uptake and intracellular ISIS 1570 distribution however, were dramatically altered by ON presentation as an ionic complex with a lipid mixture, DOTMA/DOPE (dioleoylphosphatidylethanolamine). While toxic at high concentrations, the quaternary ammonium DOTMA enhanced ISIS 1570 uptake into cells about 17-fold and activity (inhibition of ICAM-1 expression) > 1000-fold, apparently by enabling preferential transfer to the cell nucleus and cytoplasm. At 8 μM DOTMA solution and 1 μM ISIS 1570 concentrations, the cellular uptake rate was approximately 10.4 pmol of ISIS 1570/10^6 cells per h [92]. Secondly, antibody-mediated delivery of ONs has also been successfully used to not only target ONs to the desired cell, but to increase uptake (see [91]). While these studies demonstrate favorable in vitro results, considerable research is still required for effective delivery of ONs in vivo to achieve therapeutic concentrations within target cells.

3. Aerosol dosimetry

This discussion will center on delivery to the lung itself (the trachea and below) and assume that 'oral' as opposed to 'nasal' inhalation is practised because of the resultant increase in lung deposition associated with this mode of inhalation [93]).

Only a basic understanding of the factors affecting aerosol deposition in the lung is necessary in order to realize that neither the majority of continuous generators (compressed gas driven nebulizers, ultrasonic nebulizers) nor the so-called 'metered dose' devices (pressurized metered dose inhalers, MDIs; inhalation driven metered dose dry powder inhalers, DPIs) are designed so that the drug dose predominantly reaches the airways and alveoli. MDIs and DPIs limit only the amount a patient can inhale from a single use of the inhaler. The existing devices fail to standardize the fractions of the drug dose likely to deposit in the lung or to restrict deposition elsewhere. This is largely because it has not proven necessary to achieve these restrictions in the case of drugs administered to the lungs of asthmatics for their local effects. It is perfectly possible of course, to limit the output of MDIs, nebulizers and DPIs in order to exclude from their output aerosolized material which would otherwise deposit in the oropharyngeal regions [94–97].

3.1. Estimating lung deposition

If inhalation is to be used for local or systemic delivery purposes, the drug formulator and product designer must try to deliver a known dose more or less reliably to the lungs of a patient. Thus, the dose delivered to the lung itself, and its

reproducibility, becomes much more important than the metered dose emitted by the chosen inhaler. This seems especially so for ONs, where material delivered to the gastrointestinal tract (the majority of non-lung deposited drugs from metered inhalers is ultimately swallowed), will be destroyed by nuclease degradation (W.A. Lee, personal communication, Meeting of the American Association of Pharmaceutical Scientists, November 1994). While aerosol delivery to breathing-impaired subjects is difficult to reproduce, the same statement is not true of delivery to lung normal patients. Total deposition in the lungs of normal humans for well characterized particulate aerosols shows a coefficient of variation of the order of about 10% [98,99], a value which is in keeping with our concepts for reproducible dosing within the pharmaceutical sciences. These statements imply that dosing for systemic purposes can be made quite reliable (lung normal individuals), while high coefficients of variation should be anticipated for lung deposition in broncho-constricted or obstructed individuals [93].

If we assume at this stage that it is possible to generate several tidal volumes of homogeneously distributed aerosol (solid or liquid particles suspended in a gas phase) and make this available at the subject's mouth, in principle it is possible for the formulator to estimate the dose administered to the lungs (Dose to lung, DTL). Several basics should be standardized and have known values [100]. These are (a) drug concentration in air, (b) aerosol size distribution (drug mass median aerodynamic diameter, MMAD, and some measure of polydispersity, like geometric standard deviation) and (c) the subject's breathing pattern (rate and frequency of inspiration, duration of breathhold and inhaled volume). From these variables it is possible to estimate DTL per breath from

$$\text{DTL per breath} = \text{aerosol concentration} \times \text{fd} \times \text{TV} \quad (1)$$

where fractional deposition, fd, is given by a deposition model such as that described previously [93], TV is tidal volume and aerosol concentration refers to the drug concentration per unit volume of inhaled aerosol. Replicate inhalations will increase DTL in a proportional fashion. If the aerosol size and the breathing pattern are known, then it is usually possible to choose an appropriate deposition model and assign a value to mass fractional deposition, fd, for deposition in the entire lung [93]. Typically, in normal subjects, deposition in the alveoli is optimal for aerosols with aerodynamic diameters around 2–3 μm provided inhalation is slow (20–30 liter/min) [101,102]. This optimal size for alveolar deposition shifts toward smaller particles (1–2 μm) at faster inhalation rates, a fact which may be relevant to those researchers formulating powder inhalers. In part this is due to increased aerosol inertia and increased turbulence at faster rates of inhalation. Maximum deposition in the tracheobronchial or conducting airways occurs with larger particles although mucociliary clearance can remove material quite quickly from this region [93]. Thus, if we are aiming to optimize absorption, it is likely that we will also aim to optimize deposition in the lower lung regions where ciliary clearance is slow or absent. Perhaps paradoxically, the size optimum for local therapy (where the site of action may well be in the conducting airways; [101]) is believed to be similar to that for alveolar deposition because aerosol penetration enhancement in breathing-impaired subjects requires smaller, more stable aerosols (which remain in suspension longer). Ideally therefore, aerosols with MMADs in the 1–3 μm range with small degrees of polydispersity should be presented to the patient as a stationary, stable cloud of solid or liquid particles suspended in air.

Medicinal aerosols are presently far from ideal. Droplets propelled from MDIs have high inertia and tend to evaporate rapidly. Dry powders, even when successfully deaggregated from micronized materials, are often hygroscopic resulting in changes in their particle sizes in humid environments [103–105]. Furthermore, aerosol concentrations which are inhaled by patients as boli are not constant throughout a single breath [99]. For these reasons, it is difficult to make an absolute estimate of the concentration and size of a therapeutic aerosol. Thus, the approach described previously to estimate DTL is an approximation only.

3.2. Maximum doses

Table 3 (reproduced from [93]) shows some projections for aerosolized drug doses delivered to the lung as DTL per breath. These doses are not the same as metered doses, rather they represent realistic maxima, based on the authors' practical experience with a number of therapeutic and non-therapeutic aerosol generators. For short durations of time it is possible to generate quite large aerosol concentrations in terms of solid or liquid mass per unit volume of carrier gas. The difficulty lies, not in the production of aerosol, but in the production of respirable aerosol. In practice, it is difficult to generate a stable aerosol drug concentration of greater than about 1 mg/l in the respirable size range. Fortuitously, this statement is a useful rule of thumb which can be applied to the maximum respirable drug concentration achievable from any of the existing delivery systems (Table 3). High output nebulizers, when used with drugs in high concentrations in aqueous solution, have been reported with respirable output concentrations ranging from 200 to 400 μg drug/l in the absence of dilution air [96]. Somewhat higher values than these were possible with the SPAG II aerosol generator [96] with higher initial drug concentrations in the reservoir than those recommended by the manufacturer [93]. Pressurized MDIs containing up to 5% drug in suspension can generate a drug concentration in air of about 5 mg in a 1 l aerosol flume. Optimistically, up to 25% of this material (corresponding to about 1.25 mg/l) may be made small enough to reach the lung (assuming formulation in high vapor pressure propellant blends and the use of an attached reservoir device to enhance evaporative droplet size reduction prior to inhalation; [106]). Early DPIs, even though they sometimes contain metered doses as large as 20 mg [4], because of poor deaggregation behavior of their powder charge [106], succeed in delivering much less than 20 mg to the lung (1–2 mg/breath is not unreasonable, Table 3). More recently developed 'power assisted' DPIs may be improving on this value of 2 mg [107] but not by more than about twenty percent. For those readers familiar with the aerosol literature, some of the values for drug concentration in this paragraph may appear to contradict some of the quoted 'aerosol concentrations' from different generators. It is important to recognize that it is the drug we try to deliver while the aerosol is merely the carrier. Thus, the distinction between drug and aerosol concentration is extremely important.

The values presented in Table 3 are speculative. However, provided drug aerosols can be made from pure drug without toxic excipients, maximum values for DTL are limited only by the number of inhalations which the patient is prepared to draw from the device. While MDIs may have limits as low as 2–4 inhalations (1 actuation per inhalation) per administration (due to propellant induced side effects), nebulizers (containing only dissolved drug) and DPIs (containing

Table 3
Common values for dose to lung (DTL) in mg per breath for existing delivery systems alongside possible projections for what these systems may be made to achieve (reproduced with permission from Ref. [93])

Device		Typical DTL/breath	Projected DTL/breath
Small portable nebulizer (non-metered; airblast)	[a]	0.001	0.25
High output nebulizer (non-metered; airblast)	[a]	0.5	3.0
Ultrasonic nebulizer (non-metered)	[b]	0.6	3.0
Pressurized MDI (metered)		0.01–0.5	1.25
Dry powder inhaler (metered)	[b]	0.005–0.25	1.0

Projections are based on a practical maximum concentration for respirable aerosol of <approximately 1 mg/l. Actual aerosol concentrations may be much larger than this value if non-respirable material is taken into account.

[a] Based on a 3 l tidal volume or volume per breath. Portable nebulizers are presently incapable of 1 mg/l respirable aerosol concentrations. Projected values for DTL in the case of high output nebulizers are greater than those to be expected from aerosol-bolus-generating MDIs because of the nebulizer's continuous output capability.

[b] Ultrasonic nebulizers and DPIs presently have high apparent output concentrations with large quantities of non-respirable aerosol. Unlike MDIs, they are difficult to baffle in order to reduce oropharyngeal deposition.

drug alone or drug blended with inert sugar carriers) may have enormous dosing potential. The downside to the use of multiple inhalations as a means of achieving high dose drug deposition is the attendant lack of convenience to the patient; while 1–4 puffs may be tolerable, much beyond that becomes an annoyance. Table 3 can thus be used as a simple guide to assess the feasibility of delivering adequate doses for both systemic or local purposes. Typical ON parenteral doses of 0.2–20 mg/kg in human studies (W.A. Lee and S.T. Crooke, personal communication, Meeting of the American Association of Pharmaceutical Scientists, November 1994) show that for systemic delivery purposes, inhalation may be a feasible alternative to injection only under optimal conditions and provided the ON in question is fairly potent and largely bioavailable.

3.3. Formulation and stability

While there is little information in the literature on this subject, there are several important points which can be made. Unlike many protein products which may require associated solvents and/or ions for conformational stability [93], ONs usually present as amorphous, lyophilized white powders with potentially excellent purity profiles (Table 2). Solid state chemical stabilities are good and reconstitution in pure solvents (usually water) is simple and rapid. In the case of the phosphate-neutral methylphosphonates (Table 2, Fig. 1), co-solvents either may be required or have been used to enhance solubility [108]. Because these molecules carry a variable mix of bases with their respective pK_a values, the charge on the bases will depend upon the pH of the solvent (Table 4). At neutral pH, however, the bases will be uncharged and will not affect the overall charge in neutral solution. Phosphodiester ONs will carry a net negative charge due to the ionization of the phosphate backbone. Similarly, phosphorothioate ONs will also carry anionic charge which resides primarily on the sulfur at physiologic pH [109] (the pK_a of phosphoromonothioic acid is 1.49). In contrast, the backbone of methylphosphonate ONs will remain uncharged at pH 7 due to the substitution of ionizable species in the backbone with uncharged methyl groups. It should be noted that the phosphorothioate and methylphosphone backbone modifications result in the formation of 2″ stereoisomers. Stereoisomer formation results in decreased binding affinities for complementary RNA sequences, although the increase in stability of phosphorothioate ONs relative to phosphodiesters results overall in a decreased effective concentration. Methylphosphonates, in contrast, continue to require very high doses for activity and consequently are not likely to be strong candidates for aerosol delivery.

Typical molecular masses range from 5 to 7 kDa (Table 2), putting these molecules clearly in the 'oligo' nucleotide size range. Thus, the changes in solution conformation and tertiary structure (denaturation) seen with much larger 'macro' molecules are unlikely to be a problem with ONs. All of the major dosage forms are theoretically possible; metered dose inhalers with ONs in suspension in propellants, dry powder inhalers with ONs metered pure or with inert 'inactive' diluents (excipients), or ONs reconsti-

Table 4
pK_a values of mononucleotides

Mononucleotide	Base (position)	Pentose sugar	Phosphate
Adenosine 5′-phosphate	3.74 (N1)	13.1	0.9
Guanosine 5′-phosphate[a]	2.9 (N7); 9.6 (N1)	≈12	0.7
Cytidine 5′-phosphate	4.5 (N3)	≈12	0.8
Thymidine 5′-phosphate[b]	10.0 (N3)	≈12	1.6

The pK_a values of the mononucleotides which compose DNA and ONs are compared. The base, sugar and phosphate of the nucleotide each have ionizable groups whose pK_a values differ depending on the nucleotide. Data are adopted from Ref. [111].
[a]Guanosine 5′-phosphate deprotonates at N1 in alkaline media and tautomerizes resulting in a negative charge on the C6 carbonyl.
[b]Thymidine's N3 is acidic and deprotonates with increasing pH.

tuted as aqueous solutions and administered by nebulizer.

Because there are a number of texts and reviews (e.g. [100,106,110]) describing modern methods of aerosol formulation, generation and delivery, it is not our intention to describe this area in detail; rather we will discuss some of the issues as they relate to the major forms of inhalation device (Table 3). To most researchers working with water-soluble molecules (Table 2), simple jet or ultrasonic nebulizers would be the first choice for study of inhaled effects and proof of concept. The practical difficulties associated with these devices in the longer term occur in two major areas. First, the lack of device standardization and the large number of different nebulizers available in practice means that even a fixed formulation will not be administered in a consistent way, unless that formulation is co-marketed with a complete nebulizer and detailed directions for its use. Second, formulation development of sterile, isotonic aqueous drug solutions for eventual nebulization is not always as simple as it appears. For dosing reasons, it may be desirable to use quite high concentrations of ONs and, in some cases, molecular aggregate formation and binding to the device can then be a function of ON and ion concentration in solution (S. Wu-Pong, unpublished data). However, while nebulizers can denature macromolecules and proteins, this is unlikely to be a significant problem with ONs since these molecules are relatively small and lack significant tertiary structure. Overall, aqueous solutions of ONs, administered via previously characterized nebulizers, appear to be a simple means of determining the usefulness of ONs delivered by inhalation.

The reactivity of the components of the ON (base, sugar, glycosidic bond, phosphodiester bond) have been well documented (for review see [111]) and should be considered in the formulation of aqueous solutions for use with nebulizers. First, the bases may react with electrophilic reagents resulting in heterocycle halogenation, nitration, methylation, and oxidation. Reactions with nucleophilic reagents may also result in amino group substitution. Such reactions occur in basic conditions in the presence of amines. Deamination of the bases may also occur in alkali conditions. Second, reactions involving the nucleotide's sugar include substitution, acylation, oxidation, or reactions with carbonyls to yield cyclic acetals or ketals (formed between the base and sugar). Third, the glycosidic bond is highly stable under neutral or basic conditions, but is highly sensitive to acid hydrolysis. Acid hydrolysis of the glycosidic bond of nucleotides occur rapidly at pH 1.0, 37°C when the base is a purine (A or G, $k_1 = 10^{-4}\ s^{-1}$) but occurs more slowly when the base is a pyrimidine (C or T, $k_1 = 10^{-8}\ s^{-1}$). Finally, the phosphodiester bond may be cleaved by acid hydrolysis (pH <3). After acid-hydrolyzed depurination, an aldehyde forms at ribose's C1 followed by cleavage of the C-O bond at the ribose's C3 carbon then β-elimination of the phosphomonoester 3′ to the ON. The phosphodiester backbone may also be cleaved by nucleases and by oxidative degradation, but is otherwise stable at neutral and basic pH. Methylphosphonate ONs, however, are degraded under basic conditions, so synthetic schemes must avoid the basic conditions which are used in phosphodiester and phosphorothioate ON synthesis. However, although these reactions have been characterized, the reactivity of each component of the ON may differ significantly compared to the reactivity of the monomer due to steric factors associated with the primary or secondary structures of the ON.

In the longer term, smaller, more convenient inhalers (MDIs and DPIs) are much more suitable for use by ambulatory patients. While DPIs are presently popular because of the absence of propellants, the development of a reproducible inhaler (device plus formulation which functions in concert) is no easier than that of an MDI. Furthermore, in the case of an MDI, there is no reason why an ON would prove any more difficult to suspend and meter from suspension than say, the small bronchodilator, albuterol, or the peptide, leuprolide acetate [93,112]. While the formulation and packaging of MDIs and DPIs have been described in detail previously [106], it is important to note that both types of inhaler will probably be forced to employ spray-dried or freeze-dried ONs (in suspension in

liquefied gas propellants and in formulated powder blends, respectively). The consequences of powder processing, such as milling, has not been published. Nor is there information in the literature on the effects of hydrophobic propellants and the surface-active agents often employed in pressurized MDIs. However, unless there are unpredictable problems associated with the solid state chemical reactivity of ONs (for example, with excipients used in processing and formulation [113]), their chemical stability in the dry state is not likely to be a problem. Their physical stability, on the other hand, merits careful investigation in both MDIs and DPIs because ON powders are likely to be amorphous (as opposed to crystalline) and formulations are therefore likely to be sensitive to moisture. Moisture invasion into nonaqueous MDI formulations can bring about crystal growth [114,115] and amorphous materials in suspension are known to be prone to this problem [116]. Clearly, the result of crystal growth in an MDI formulated as a suspension can either be an unusable product or, perhaps, one in which dosing uniformity is a problem [117]. Amorphous powders can also pose problems in DPIs where the formulation will probably need to be protected from sorbed moisture. Moisture sorption at high relative humidities can result both in solid state recrystallization and/or in large increases in interparticulate cohesion forces, thus preventing successful aerosolization [118].

4. Pulmonary absorption

In the lung-normal individual, where aerosol deposition is quite reproducible [93], there is reason to believe that there are fewer complications likely to interfere with the absorption process than, for example, there exist in the gastrointestinal tract. In this respect inhalation may offer fairly reproducible absorption kinetics and thus have some advantages. Moreover, it is unlikely that this route would be employed for systemic delivery purposes in patients with lung disease. Obvious differences between the lung and the intestine include the lack of dietary complications in the lung, the low levels of extracellular enzymes [119,120] and the absence of quantitative and qualitative inter-individual metabolic differences due to the presence of different flora and fauna.

4.1. Macromolecular absorption determinants

The pulmonary bioavailability of macromolecules is largely controlled by the relative rates of several competing clearance processes (to sites other than the systemic circulation). These are, quite simply, the lung's mucociliary escalator (clears material from the upper conducting airways, via the larynx, to the gut), lung lymphatics (which clear material very slowly from the lower airways or alveoli), lung metabolism and lung binding [121]. All of these processes compete kinetically for absorption. Presently, there is negligible information on lung metabolism or binding of ONs although conventional wisdom shows that only lipophilic proton-accepting weak bases (this excludes most negatively charged ONs) are significantly, but reversibly, bound to lung [121]. Moreover, while binding may have ON structure-specific affinities, it is likely only to slow down absorption, not to prevent it. Nucleases, on the other hand, which are responsible for ON breakdown in vivo [20,53,54] are, like proteases, ubiquitous in the body. Thus, whether ONs are absorbed intact through the lung will depend upon the concentration and location of these nucleases and the mechanisms of ON absorption. Metabolic studies with ONs remain to be performed in lung and will, no doubt, show ON-structure-dependent results.

Both the conducting airways and the alveoli are sufficiently permeable to show significant ON absorption [122]. The alveolar regions are likely to be the most important however, because clearance is considerably slower from the periphery of the lung than it is from the upper airways [101,123], and absorption of molecules in this molecular size range is normally quite slow. Variable upper airway deposition, in the case of slowly absorbed macromolecules, can result in variable bioavailabilities because of the significance of clearance from the upper airways to the gut [2,124]. For larger molecules therefore, targeting the lower airways to increase the dura-

tion of residence in the lower lung, should lead to a greater opportunity for absorption from the alveoli.

The various physicochemical factors which control pulmonary absorption kinetics have been reviewed previously [2,121,123]. Suffice it to say that the epithelial barrier will be rate-determining in the absorption process for these compounds and that the important information concerning ONs should relate to the effects of molecular charge on compounds in the 5–7 kDa size range. Table 5 reviews the literature data in this area and some new information from our laboratory on electronic charge effects ([125] and Z. Sun, P.R. Byron, F. Rypacek, unpublished data). All the macromolecules in Table 5 are effectively oligomers with somewhat different shapes in solution. They may be absorbed either via tight junctions in the epithelial cell layer or by transcytosis, or both ([125]; diffusion through lipid cell membranes is extremely unlikely). While the comparative absorption (relative to injection) of a variety of polypeptides has been reported from the rat lung following solution instillation [2,126,127], absorption kinetics were not studied in those cases and most compounds were outside of the size range of interest (5–7 kDa; Table 2). In contrast, Table 5A shows the

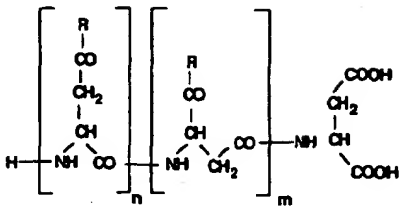

Fig. 5. Structure of synthetic polypeptides. Poly-a,b(*N*-(2-hydroxyethyl)-DL-aspartamide). PHEA [R=-NH-C_2H_4OH] and its two random co-polymers PHEA-DMAPA [12% of R=-NH-C_3H_6-$N(CH_3)_2$] and PHEA-AA [16% of R=-OH]. For analytical purposes, fluorophore replaces 0.3% of hydroxyethyl groups on PHEA to give F-PHEA (Table 5) in all cases.

alveolar absorption of a series of hydrophilic synthetic polypeptides which are not metabolized by the perfused lung [125]. The compounds are based upon fluorophore-labeled poly-hydroxyethyl-aspartamide, F-PHEA, prepared from a racemic mixture of DL-aspartic acid, via polysuccinimide, to give random α,β linkages (Fig. 5). The positively charged dimethylaminopropyl aspartamide (DMAPA) occupied 12% of the monomer subunits while the negatively charged aspartic acid (AA) was 16% with respect to the monomer. Both were random copolymers with F-PHEA and carried the charge as a function of

Table 5

(A) Mean absorption of ON-sized, polydispersed linear macromolecules with different charges (Fig. 5) in the isolated perfused rat lung (weight mean molecular masses of absorbed materials were approximately 7 kDa; nominal doses of 1 mg/0.1 ml were administered as coarse aqueous sprays phased with inspiration [125])

Macromolecule	Charge	Percent absorption after		
		30 min	60 min	120 min
F-PHEA[a]	neutral	15.9	22.3	29.2
F-PHEA-DMAPA[a]	+ve[b]	15.1	18.8	23.4
F-PHEA-PAA[a]	−ve[b]	23.9	32.7	42.8

(B) Pulmonary absorption of 'ON-sized' inulin and insulin from the lungs of the intact rat and rabbit, respectively. Data is calculated[c] for comparative purposes, based on the absorption half-lives [133,139]

Macromolecule	Molecular mass (kDa)	Percent absorption after		
		30 min	60 min	120 min
Inulin	5.25	8.8	16.9	30.9
Insulin	5.8	28.1	48.3	73.3

[a]See Fig. 5. Random co-polymers containing approximately 12% DMAPA or 16% PAA, respectively.
[b]Net charge assumes ionization at physiologic pH.
[c]Percent absorption = [1 − exp(− absorption rate constant × time)] × 100.

the protonation of the amine (F-PHEA-DMAPA) or deprotonation of the carboxylic acid (F-PHEA-AA) at physiologic pH. Studies were conducted in the isolated perfused rat lung following effective delivery of approximately 80% of the nominal 1 mg dose to the alveolar regions of the lung preparation. Overall, and irrespective of charge, some 18–33% absorption occurred in the first hour following administration. The lack of difference between results for the neutral and positively charged compounds may imply the importance of transport via tight junctions for these molecules (adsorptive endocytosis is believed to be enhanced for positively charged species passing by this mechanism, due to the negative charge on the apical surface of epithelia). This remains to be proven however, and the influence of charged lung surfactant molecules is unknown. The enhanced, but more variable transfer of the negatively charged polyionic F-PHEA-AA is probably due to the membrane toxicity of these anionic species [128] which may 'enhance' their own absorption at these relatively high concentrations. Despite the fact that we know that F-PHEA has dose-and/or concentration-dependent absorption [125], these results overall are in good agreement with the work of Schanker's group on the 5.25 kDa polysaccharide, inulin (Table 5 [129]) in the in situ rat lung. The 5.8 kDa poly-zwitterion, insulin, has more structure in solution and is known to be well absorbed following aerosol administration to the human [2]. Recent results, showing quite rapid insulin absorption in the rabbit (Table 5 [124]), demonstrate the possibility of species and inhalation mode-dependent results. Some projections from that study [124] were included in Table 5 for comparative purposes and to illustrate the need to study absorption of each molecule of interest, under conditions which are likely to mimic those seen during therapy.

In the absence of specific ON absorption studies in lung at this time, we must make assumptions if we are to answer the questions posed at the outset of this article. Since polypeptides behave similarly to ONs in solution in terms of polyionic and hydrogen bonding properties, ONs and proteins should also behave similarly in terms of lung absorption. Thus, assuming that approximately 20% of a 5–7 kDa ON can be absorbed in the first hour after its administration by aerosol (based upon results for F-PHEA and its co-polymers in Table 5), and that absorption is apparent first-order, this result would correspond to an absorption half-life of approximately 3 h. The further implication of this statement, that it will take some 4.3 half-lives, or 12.9 h to effect 95% absorption, shows the importance of deep lung aerosol dose deposition. Competing clearance of material deposited in the upper airways (half-life $\approx$1 h [101]) is predictably much faster than absorption in this molecular size range.

5. Example case study: ICAM-1 receptor suppression in lung

While systemic administration of ONs via the lung requires careful selection of both ON and inhaler technology, aerosol delivery for local therapeutic purposes promises to be much easier. Section 2 of this paper addressed several of the different types of ONs and disease processes which may be treatable by ON-induced regulation of gene expression. That discussion was limited by the paucity of information concerning the differences in biochemical mechanisms and potential ON targets in healthy and diseased lung (section 2.2). This is a fertile research area and, as the cell biology of lung disease processes unfolds in the literature, local antisense targets will increase in number and variety.

5.1. Selection of ON and 'mode of action'

Because this section is intended to illustrate a series of 'thought experiments' leading to a potential in vivo experimental starting point, we began by searching for an appropriate therapeutic target. This could be a protein, expressed in the alveolar airspaces or on the apical surface of airway epithelia such that its over-expression results in pathologic consequences. While we do not preclude the choice of a target in the lung interstitium or endothelium, the chosen ON must reach an intracellular site of action or 'biophase'.

Furthermore, cellular uptake of ONs is known to be a problem (section 2.3.2). It follows then, that uptake should best be enhanced by maintenance of high local concentrations proximal to the cell in question; hence the choice of a cell target for aerosol therapy which presents on the airway side of lung epithelium. The data shown for PHEA in Table 5 and earlier discussions suggest that ON alveolar airway concentrations in the 3 h or more following aerosol deposition will exceed 50% of their initial concentrations (note that when ON aerosols are to be used for local purposes, systemic absorption becomes a clearance process which defeats our objectives and demands larger doses).

Many lung diseases are either caused by inflammation or have inflammatory processes associated with their pathogenesis ([1] and section 2.2.1). This has lead to considerable drug company research for improved anti-inflammatory molecules like steroids and antileukotrienes [1]. Quite recently, research on intercellular adhesion molecules (ICAMs) has shown that ICAM-1 is involved, not only in lymphocyte and neutrophil-induced pulmonary edema via its association with pulmonary endothelial cells [130,131], but that the ICAM-1 receptor is expressed at much higher levels on the luminal surface of alveolar type I epithelial cells during experimental inflammatory injury [132]). ICAM-1 is a 90–110 kDa membrane glycoprotein which is involved both with the movement of leukocytes out of blood vessels and with antigen presentation to lymphocytes [133,134]. Moreover, alveolar type I cells cover 95% of the alveolar surface area and are essentially responsible for functional gas exchange [135]. Because the ICAM-1 receptor is known to be causally involved in the pathologic accumulation and attachment of leukocytes via their own ligands, the β-2 integrins, it is entirely logical to attempt to down regulate ICAM-1 expression in the airways of individuals suffering from inflammatory lung diseases.

Fortunately for this example, a group of researchers at ISIS Pharmaceuticals published a classic paper in 1992 [92] on the cellular uptake of phosphorothioate antisense ONs in vitro as described in section 2.3.2. Their paper, upon which we will base the remainder of this example case study, concerned the uptake and intracellular distribution of phosphorothioate ON ISIS 1570 and the resultant inhibition of ICAM-1 expression on HUVEC (human umbilical vein endothelial cells). ISIS 1570, according to Bennett et al. [92], is an 18-nucleotide ON (molecular mass 6178 Da), which retains its polyanionic structure (Table 2), but has decreased sensitivity to nucleases compared to phosphodiester ONs. Provided it can penetrate the cell membrane (section 2.3.2) this ON is expected to block ICAM translation and induce degradation of the target mRNA via an RNase H-dependent mechanism.

5.2. Formulation and delivery

The use of cationic lipids and other vectors to enable and enhance the cellular uptake and activity of ONs has been briefly discussed in section 2 and reviewed by other authors [92,136–139] in this and other journals. However, the mode and formulation for optimal uptake enhancement is likely to be compound specific. We mention them here because it appears to be necessary to use vectors of one form or another, to deliver both genes (polynucleotides) and ONs to their intracellular targets. Loose associations of ONs and excipients like DOTMA can dissociate following administration. However, local administration by aerosol is a good deal less challenging than injection in this respect (where passage through the body involves considerable dilution). Indeed, it is likely that cell-uptake enhancement in the lung could be quite effective, simply by co-administration of ON with a suitable excipient [139].

To continue with ISIS 1570 as our example, let us assume that this molecule is mixed with the optimal [92] 1:8 molar ratio with DOTMA as our formulation. Provided that ICAM-1 expression is inhibited optimally in alveolar type I cells at a similar concentration to that used with HUVEC cells (1 μM ISIS 1570), dose (DTL, section 3) calculation for alveolar deposition is a relatively simple matter. If the minimum thickness of the solution coating the expanded alveoli was no more than the thickness of a monolayer of lung surfactant (of the order of 25 Å or 2.5×10^{-9} m),

and the expanded human lung had a surface area (primarily alveolar) of about 75 m^2 [135], the product, thickness×area, would give a minimum volume coating the alveoli of only 1.875×10^{-7} m^3 or 0.1875 ml. While this value is clearly too small [101], especially in the case of inflamed lungs, 25 ml is probably a reasonable upper limit for the purposes of our calculation. Thus, to obtain a 1 μM drug solution, a maximum deposited dose of 154 μg ON (with approximately 250 μg DOTMA) would be required. Because Bennett et al. were also able to obtain considerable inhibition with 10-fold lower ISIS 1570 concentrations, this is probably more than is truly necessary as a starting point in both animal or human phase 1 dose ranging studies. Importantly however, the lung delivery of these types of doses is well within the range of any of the existing delivery systems (Table 3) and none need be excluded. Indeed, we advocate a test of ON feasibility by inhalation aerosol based upon these calculations. Such tests must include the impact of the aerosol formulation on the properties of the lipid-ON complex. Variables such as ionic strength and buffer composition may influence the size and shape of the polyanionic ON and cationic lipid complex. How this will control the final disposition of these complexes after deposition is a subject ripe for further research.

6. Concluding remarks

In this review, the state of the art in ON and aerosol technology were reviewed to give the reader an overview of how each will impact on the formulation and use of ONs for aerosol delivery to the lung. Several salient points were also illustrated in this communication. First, the maximum drug doses which can be administered through the lung (Table 3) may limit the use of the lung to systemically deliver ONs, since current minimum ON human doses represent maximum drug doses which may be delivered systemically through the lung under ideal conditions assuming 100% bioavailability. This finding, however, does not preclude the use of ONs to directly treat lung disorders by delivering therapeutic ON doses directly to affected tissues. Second, currently used ONs (molecular mass 5–7 kDa) are likely to be compatible with pulmonary absorption based on information available for other macromolecules of similar molecular mass. Such data (Table 5) suggest that absorption of 1–2 mg every 8 h could be a feasible dosing regimen for these compounds based on projected absorption profiles. Third, in vitro and in vivo animal studies demonstrate that cellular uptake or delivery of ONs to the cytoplasm or nucleus may be enhanced using drug carriers or viral vectors. Since currently used ONs are limited by in vivo stability and cellular uptake, such formulations may be necessary to provide therapeutic doses of ON in quantities which may be delivered via aerosols. Finally, while aerosol formulation and generation problems are likely to be encountered during testing of ON delivery to the lung, ONs may present fewer problems compared to their protein counterparts. Initial work in this area should begin ON delivery *via* nebulizers to demonstrate the feasibility of delivering ONs to the lung, followed by developing metered dose or dry powdered formulations as line extensions in product development. In conclusion, while ON administration to the lung is limited in some areas, such as systemic delivery, ON delivery to the lung may prove a feasible approach to specifically regulate the genes associated with respiratory infection, cancer, or pulmonary inflammatory diseases.

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